Epigenetic regulatory pathways involving micro-RNAs may modulate the host immune response following major trauma

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Running head: MicroRNAs and immunosuppression in trauma patients

Abstract

Background: Post traumatic nosocomial pneumonia is a common complication resulting in significant morbidity. Trauma-induced immunocompromise is associated with an enhanced susceptibility to pneumonia. In this study we explore the hypothesis that post-transcriptional epigenetic regulation of gene expression may be an important factor in determining this immune phenotype. We describe the pattern of production of micro RNAs (miRs) and their association with nosocomial pneumonia following severe trauma.

Methods: A convenience sample of 30 ventilated polytrauma patients (UKCRN ID: 5637) and 16 healthy controls were recruited. Messenger RNA (mRNA) and protein levels of key cytokines were quantified within two hours of the injury and at twenty-four hours. Three miRs per cytokine were then selected based on miRBase target prediction scores and quantified using polymerase chain reaction. Nosocomial pneumonia was defined using the Center for Disease Control and Prevention definitions.

Results: Median injury severity score was 29 and 47% of patients developed nosocomial pneumonia. miR-125a and miR-202 decreased by 34% and 77% respectively immediately following injury whereas their target, IL-10, increased mRNA levels 3-fold and protein levels 180-fold. Tumor necrosis factor alpha (TNF-α) and IL-12 gene expression decreased by 68% and 43% respectively following injury and this was mirrored by a 10-fold increase in miR-181, a miR predicted to target TNF-α transcripts. Lower levels of miR-125a and miR-374b were associated with the later acquisition of hospital-acquired pneumonia.

Conclusions: Alteration in the expression of miRs with highly predicted complementarity to IL-10 and TNF-α may be an important mechanism regulating the post-traumatic immunosuppressive phenotype in ICU patients.

Level of Evidence: Retrospective observational study, level III.

Key words: Epigenetics • miRNA • IL-10 • Immunosuppression • Polytrauma • Pneumonia

Background

Trauma remains a frequent cause of death and results in an annual global death toll in excess of 6 million.[1] Although the early mortality rates continue to fall with improved pre-hospital care and more structured trauma systems, the medium and long-term mortality rate in those patients that survive to require treatment in an intensive care unit (ICU) remains high.[2] Severely injured patients are highly susceptible to hospital-acquired infection, particularly pneumonia. The development of sepsis in trauma patients is usually preceded by a characteristic cytokine profile[3, 4] and is associated with a 3-fold increase in mortality.[5] We have previously described a hyper-acute immune response to severe trauma, dominated by excess interleukin 10 (IL-10) gene expression that is associated with the later development of infectious complications.[6] This immune response is further characterised by features of impaired innate immunity and T helper cell type 1 (T_h1) responses with marked reductions in tumour necrosis factor-alpha (TNF-α) and IL-12 gene expression.[7]

The coordinated production of miRs represents a key epigenetic mechanism regulating gene expression through either transcriptional repression or messenger RNA (mRNA) degradation.[8] miRs are short, single-stranded RNA molecules that bind to a target mRNA through sequence complementarity at the 3 prime untranslated region (3' UTR) of the mRNA and are involved in the transcriptional regulation of 60% of human protein encoding genes.[9] This mechanism of action has facilitated the development of bioinformatics tools that determine which miRs are predicted to target specific mRNAs based on their base-pair sequence.[10]

As genetic association studies of the prototypical anti-inflammatory cytokine, IL-10, and sepsis susceptibility have provided inconsistent results,[11] we hypothesised that other regulatory mechanisms may be of greater importance in determining gene expression following trauma. In

this study we assay micro RNAs (miRs) predicted to target IL-10, TNF-α and IL-12 following severe trauma in order to explore the hypothesis that changes in cytokine gene expression may be coupled with changes in the expression of those miRs predicted to target them, thereby suggesting a regulatory role in this setting.

Methods

This study was conducted at a major tertiary referral trauma center, The Royal London Hospital, UK. The study was approved by the East London and City Research Ethics Committee (07/Q0603/29). Deferred informed written consent was obtained from each patient or their next of kin.

Patient selection

All adult trauma patients (>15 years) who met the local criteria for trauma team activation were eligible for enrolment into the Activation of Coagulation and Inflammation in Trauma (ACIT) 2 study when research personnel were present (8am to 8pm daily). ACIT2 is a study prospectively evaluating aspects of coagulation and inflammation in trauma patients (UKCRN ID: 5637). Those patients who were transferred to the ICU following their initial resuscitation and treatment were eligible for inclusion to this study.

Exclusion criteria included; arrival at hospital more than 2 hours after injury, extensive burn injury, HIV infection, immunosuppression secondary to chemotherapy or corticosteroids.

In the original studies 112 patients were available for analysis.[6] For this study, 30 patients remained with a suitable quality and sufficient quantity of total RNA available for miR analysis. Therefore, this study is the analysis of a convenience sample of patients for whom stored, viable samples were available from the original cohort of 112 ACIT2 patients.

16 healthy subjects were recruited from laboratory and hospital staff to comprise a control group.

Data collection

Extensive clinical, demographic and injury specific data were collected and pneumonia was defined as per CDC guidelines as previously described (Supplementary Table 1).[6, 12] Adjudication of infectious complications was performed in a blinded manner independently of the clinical team (by MJO'D and HDTT).

Blood sampling

Blood samples were taken on arrival in the emergency department within 2 hours of the trauma and at 24 hours after admission. Blood was collected into a PAXGene^{TM} blood RNA tube (PreAnalytix, Germany). Plasma was collected from a citrated vacutainer (Becton Dickenson, UK) centrifuged twice at 3,400 RPM for 10 mins and stored at -80^{o}C .

RNA quantification

Total RNA was extracted from a whole blood sample collected in a PAXgene tube, reversed transcribed to cDNA and both mRNA and miR quantified using quantitative real time polymerase chain reaction (qRT-PCR) as previously described.[6, 13] Relative quantification was calculated using the standard delta-delta methodology. Results were expressed as a normalized ratio of candidate gene to reference gene for both mRNA and miRNA analysis.

Enzyme Linked Immunosorbent Assay (ELISA)

Samples were assayed in duplicate using commercially available high sensitivity ELISAs (Life Technologies, Carlsbad, CA). Absorbance was measured at 450nm.

Selection of miRNAs

We have previously described three genes, IL-10, TNF-α and IL-12, whose expression levels change following severe trauma and relate to infectious complications.[6, 7] In order to select miRs that could target IL-10, TNF-α, and IL-12, a bioinformatics search was performed utilising the *TargetScan*[14] and *microRNA.org*[15] databases. Only miRs which were conserved in mammals, and predicted by both *microRNA.org* and *TargetScan* were selected for this study. Based on the mirSVR score produced by *microRNA.org*,[10, 15] the top three miRs targeting IL-10, TNF-α or IL-12 were selected for further analysis (Supplementary Table 2). mirSVR is a regression model that is trained on both the sequence and contextual features of the predicted miRNA:mRNA duplex, and mirSVR downregulation scores are calibrated to correlate linearly with the extent of downregulation. The predicted targets for each miR are represented as Supplemental Digital Content in Supplementary Table 2.

Statistical analysis

All statistical tests are two-sided with *P*-values of $P<0.05$ considered significant and are reported without correction for multiple comparisons. Differences in categorical variables were calculated using a Chi-squared or Fisher's exact test as appropriate, and the Wilcoxon rank sum test for continuous variables. The Wilcoxon signed-rank test was used to analyse serial samples. Spearman's rank correlation coefficient was used to describe correlation. Data analysis was performed using the JMP (version 11) statistical software (SAS, Cary, NC, USA).

Results

Patients

A total of 112 ICU patients with severe traumatic injury as an admission diagnosis were enrolled to ACIT2 between September 2010 and October 2012. Gene expression patterns in this group have been described in detail elsewhere.[6, 7, 16] For this study, we analysed the 30 patients that remained with suitable quality and a sufficient quantity of RNA available for miRNA analysis. These patients were recruited between September 2010 and July 2012. Demographic and clinical details of this cohort are shown in Table 1. 14 (47%) patients developed a clinically defined pneumonia (Supplementary Table 1)[12] during their hospital admission. Microbiological characteristics of these infections are outlined in Table 2. Pneumonia occurred a median of 3 (IQR 2.75-6.25) days following hospital admission. 6 (20%) patients died prior to hospital discharge. 16 healthy control samples were also collected. Median age (31.5, IQR 28.25-37) and sex distribution (62.5% male) in the control group was similar to that observed in the study patients.

Changes in miRNAs expression following to severe trauma

IL-10 mRNA levels increased 3-fold at baseline when compared to healthy controls (P <0.0001) and then increased a further 4.5-fold at 24 hours (P<0.0001; Figure 1A). IL-10 protein levels were also markedly increased at baseline when compared to healthy controls (P<0.0001; Figure 1B). At 24 hours, IL-10 protein levels fell compared to baseline (P<0.05), but remained higher than IL-10 protein levels in healthy controls (P<0.0001). The top three miRs predicted to target IL-10 mRNA were then selected (see methods) and quantified. Levels of miR-202 decreased immediately following trauma (P<0.0001 controls versus baseline) and then increased

between baseline and 24 hours (P=0.0035). However, levels at 24 hours remained less than controls levels (P=0.009; Figure 1C). Levels of miR-125a decreased immediately following trauma (P=0.02 controls versus baseline) and were then unchanged at 24 hours (Figure 1D). Levels of miR-374b immediately following trauma were not different from controls and were unchanged at 24 hours.

TNF-α mRNA levels decreased immediately following trauma when compared to controls (P<0.0001) and were then unchanged at 24 hours (Figure 2A). TNF-α protein levels were below the level of detection in the majority of patients and controls. Three miRNAs predicted to target TNF-α mRNA were then analysed. Levels of miR-181 increased immediately following trauma (P<0.0001) and then increased further at 24 hours (P=0.046; Figure 2B). Levels of miR-454 decreased immediately following trauma (P=0.0055, control versus baseline) and levels then increased at 24 hours ($P=0.02$, 24 hours versus baseline; Figure 2C). Levels of miR-301a were undetectable in all patient and control samples.

IL-12 mRNA levels decreased immediately following trauma when compared to controls (P=0.0005) and then decreased further at 24 hours (P<0.0001; Figure 3A). IL-12 protein levels were below the level of detection in the majority of patients and controls. Three miRNAs predicted to target IL-12 mRNA were then analysed. Levels of miR-410 were unchanged immediately following trauma when compared to controls and were also unchanged at 24 hours (Figure 3B). Levels of miR-21 were decreased immediately following trauma in comparison to controls (P=0.0035) and then increased over the first 24 hours (P=0.045) such that levels at 24 hours were not different from control values (Figure 3C). miR-590-5p was undetectable in the majority of patient and control samples analysed.

None of the miRNAs studied were associated with the ISS or the degree of shock as determined using the admission base deficit value as a surrogate or with the volume of crystalloid or colloid infused over the first 24 hours. Associations were observed between the number of units of packed red blood cells (PRBCs) transfused over the initial 24 hours and miR-202 levels on admission (P=0.003) and at 24 hours (P<0.0001). The number of units of both platelets (P=0.0005) and FFP (P<0.0001) transfused over the initial 24 hours were also associated with miR-202 levels at 24 hours. Supplementary Table 3 presents the univariate associations between miRNAs and other clinical and demographic variables.

miRNA expression and pneumonia

Lower levels of miR-125a at 24 hours (P=0.015, Figure 4A) and lower levels of miR-374b at baseline (P=0.005, Figure 4B) were associated with the later onset of pneumonia. No other miRNAs were associated with pneumonia. Pneumonia was not associated with clinical, demographic or injury-specific variables (Table 1).

In this cohort no statistically significant association was observed between IL- 10 levels and pneumonia (Figure 4C).

Power analysis

In the case of variables where no temporal change was detected a retrospective power analysis was performed using the mean and standard deviation obtained from the baseline values. For miR-374b and miR-410 the study was powered at 80% in order to detect a 35% and 70% difference respectively between miR levels in control samples and in the baseline trauma sample. In the case of miR-125a and TNFα the study was powered at 80% in order to detect a 58% and 26% change respectively in levels between baseline and 24 hours.

Discussion

In this study we have assayed the post-traumatic expression levels of a panel of miRs in order to determine whether their pattern of expression is consistent with a possible regulatory role in the development of the immunocompromised phenotype observed following severe trauma in ICU patients. Our analysis demonstrates that miRs predicted to target the prototypical anti-inflammatory cytokine, IL-10 (miR-125a and miR-202) decrease following severe trauma whilst IL-10 mRNA and protein levels increase. Contrasting this is the decrease in gene expression of key pro-inflammatory cytokines: TNF-α, essential for a robust innate immune response and IL-12, a T_h1 polarising cytokine. A miR predicted to target TNF-α, miR-181, demonstrates a reciprocal response and increases markedly over the initial 24 hours following severe trauma. Furthermore, lower levels of two of the miRs predicted to target IL-10 mRNA were associated with the acquisition of hospital acquired pneumonia.

miRs regulate gene expression through RNA interference. The seed region at residue 2 to 7 of the miR binds to the 3' UTR of the target mRNA which either causes degradation of the mRNA transcript or impairs efficient translation.[17] Therefore, in order to infer a possible regulatory role in the inflammatory response to severe trauma we hypothesised that miRs and their predicted target genes may change in a reciprocal manner. In our experiments we could successfully amplify miR transcript in 7 of the 9 candidates. All three assays used to quantify miRs that target IL-10 mRNA transcripts amplified successfully and 2 of these, miR-202 and miR-125a, changed in a manner that was reciprocal to IL-10 gene expression. It is interesting that although mRNA levels of IL-10 continue to increase from baseline to 24 hours, the associated protein product increases in tandem only immediately following severe trauma and then falls slightly at 24 hours. It is biologically plausible that the continued increase in miR-202

and miR-125a over 24 hours could partially account for the failure of increasing levels of IL-10 mRNA to translate into a viable protein product. In the case of TNF-α, two of the three miRs predicted to target transcripts amplified and one of these changed in a fashion that was reciprocal to the mRNA changes following severe trauma. In the case of IL-12, neither of the two miRs that amplified changed in a reciprocal pattern to the IL-12 mRNA levels. Further analysis of the potential influence of miRs on translation to the TNF-α and IL-12 protein product was limited by the sensitivity of the protein assay in clinical samples.

The expression levels of a number of the candidate miRs suggested by the bioinformatics tools did not change following severe trauma in a pattern that was suggestive of a regulatory role in this setting. Although some of these miRs have previously been implicated in immune disorders,[18-20] we could not infer a similar immune modulating role in ICU patients admitted following severe trauma. However, regulation by miRs is known to be cell state and type specific and our methodology of extracting RNA from whole blood may miss important interactions at a cellular level. In addition, the strategy of searching for reciprocal changes in mRNA and miR levels will not describe a regulatory function mediated through impaired translation as opposed to mRNA destruction. Particularly in the case of miR-410, this study may be underpowered to detect smaller changes in expression levels that may yet be of biological significance.

There remains a paucity of *in vivo* human data on the role of miR regulation of gene expression following severe trauma. One small sequencing study described 69 miRs that were differentially expressed between healthy controls and trauma patients requiring blood transfusion.[21] Of particular interest was their finding that miR-181 may have a regulatory role in the TLR signalling pathway. This may be consistent with the large increase in miR-181 expression observed in our cohort in conjunction with the decrease in TNF-α gene expression.

To our knowledge there are currently no other *in vivo* data describing miR production following severe traumatic injury in humans.

We also observed an association between decreased levels of two of the miRs that were predicted to target IL-10: miR-125a and miR-374b, and the later occurrence of nosocomial pneumonia. Whilst pneumonia was diagnosed earlier and more frequently in this cohort than in other trauma cohorts[3] the mean ISS recorded our patient cohort was also higher proportionately higher (31 vs. 19). It is plausible that decreased levels of these miRs could promote excess gene expression following severe trauma and thereby increase susceptibility to pneumonia. Although this study may be underpowered to detect an association between IL-10 levels and pneumonia we have previously reported this association in a larger cohort[7] and suggested that trauma-induced IL-10 production induced an immunosuppressive environment which increased susceptibility to nosocomial infections. Here, we suggest that this response may be regulated through miR production.

However, the observations presented here should be viewed as hypothesis generating. There are clear limitations to the utilisation of an algorithm-based program to predict mRNA-miR interactions, due to the complex interactions between miRs and their target and the redundancy in terms of the numerous putative miR recognition sites in mRNAs. Furthermore, the relatively low sample size increases the possibility of a type II error, particularly in relation to the association between age and pneumonia.

In this study we have identified three miRs, miR-202, miR-125a and miR-181, whose pattern of expression suggest that they may play a regulatory role in the immunocompromised phenotype that is observed following severe trauma and two miRs, miR-125a and miR-374b, that

were associated with the later acquisition of hospital acquired pneumonia. Additional studies will be required to validate these results and clarify the mechanisms involved.

Competing interests

The authors declare that they have no competing interests.

Author Contribution

H.C.O., H.D.T.T., K.B., R.M.P., C.J.H., & M.J.O'D. contributed to the study design and data analysis. H.D.T.T., H.C.O. and T.F.J. performed the data collection. H.C.O., T.F.J. & H.D.T.T. performed the laboratory analysis. All authors contributed to the preparation of the manuscript.

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Table 1. Patient characteristics

	Pneumonia *(n=14)*	No Pneumonia *(n=16)*	*P* value	All Patients *(n=30)*	ACIT2 *(n=112)*
Age, median (IQR)	48 (34-59)	33 (26-43)	*0.067*	41 (30-53)	41 (29- 57)
Male Sex, n (%)	11 (79%)	12 (75%)	*0.490*	23 (77%)	89 (79%)
ISS, median (IQR)	31 (23-44)	25 (19-43)	*0.465*	29 (20-44)	29 (20-36)
AIS/Head & Neck, median (IQR)	2.5 (0-3)	0.5 (0-4)	*0.776*	1.5 (0-4)	3 (0-4)
AIS/Face, median (IQR)	0 (0-1)	0 (0-2)	*0.411*	0 (0-2)	0 (0-1)
AIS/Thorax, median (IQR)	4 (3-4)	3 (1-4)	*0.097*	4 (3-4)	3 (2-4)
AIS/Abdomen & Pelvis, median (IQR)	2 (1-3)	2 (0-3)	*0.518*	2 (0-3)	0.5 (0-3)
AIS/Extremity, median (IQR)	2 (1-3)	2 (0-3)	*0.763*	2 (0-3)	2 (0-3)
AIS/Tissue, median (IQR)	0 (0-0)	0 (0-0)	*0.523*	0 (0-0)	0 (0-0)
TBI, n (%)	5 (36%)	7 (44%)	*0.722*	12 (40%)	56 (50%)
Blunt Injury, n (%)	13 (93%)	14 (88%)	*>0.99*	27 (90%)	101 (90%)
Penetrating Injury, n (%)	1 (7%)	2 (13%)	*>0.99*	3 (10%)	11 (10%)
Admission Base Deficit, median (IQR) mEq/L	6.7 (4.2-11.9)	3.7 (0.9-7.9)	*0.212*	5 (2.5-9.1)	4.1 (1.9-7.3)
Admission Lactate, median (IQR) mmol/L	3.6 (2.6-5.9)	2.9 (2.1-6.1)	*0.452*	3.4 (2.4-5.4)	3.2 (1.6-5)
PRBC, median (IQR) (Units, pre-0hr sample)	0 (0-1)	0 (0-1)	*0.901*	0 (0-1)	0 (0-0)
Crystalloid, median (IQR) (ml, pre-0hr sample)	400 (0-813)	375 (0-500)	*0.367*	400 (0-563)	100 (0-500)
HTS, median (IQR) (ml, pre-0hr sample)	0 (0-0)	0 (0-0)	*0.833*	0 (0-0)	0 (0-0)
PRBC, median (IQR) (Units, 1st 24hr)	4 (3-9)	3 (0-11)	*0.543*	4 (1-9)	4 (0-8)
Massive Transfusion, n (%) (≥10units/24hr)	3 (21%)	4 (25%)	*0.820*	7 (23%)	21 (19%)
Fresh Frozen Plasma, median (IQR) (Units, 1st 24hr)	5 (0-10)	1 (0-8)	*0.543*	3 (0-9)	0 (0-6)
Platelets, median (IQR) (Pools, 1st 24hr)	0 (0-2)	0 (0-1)	*0.926*	0 (0-1)	0 (0-1)
Cryoprecipitate, median (IQR) (Pools, 1st 24hr)	0 (0-2)	0 (0-2)	*>0.99*	0 (0-2)	0 (0-2)
Crystalloid, median (IQR) (ml, 1st 24hr)	3100 (1795-5339)	2400 (1625-5763)	*0.519*	2775 (1795-5550)	2907 (1995-4518)
Colloid, median (IQR) (ml, 1st 24hr)	1500 (688-3000)	1375 (563-2000)	*0.491*	1500 (688-2000)	1100 (489-2000)
HTS, median (IQR) (ml, 1st 24hr)	0 (0-88)	0 (0-0)	*0.687*	0 (0-0)	0 (0-0)
Ventilator Days, median (IQR)	13 (5-21)	3 (1-7)	*0.018*	6 (2-14)	4 (1-11)
MODS, n (%)	9 (64%)	6 (38%)	*0.157*	15 (50%)	43 (38%)
28 day mortality, n (%)	2 (14%)	4 (25%)	*0.490*	6 (20%)	19 (17%)

Data are expressed as median and interquartile range or absolute counts with percentages in parenthesis. The ACIT2 column represents data from the full cohort of patients from which the patients in this study originated and are described in detail elsewhere [6]. These data are provided for the purposes of comparison. *AIS*, Abbreviated injury score. *HTS*, Hypertonic Saline. *ISS*, injury severities score. *MODS*, multiple organ dysfunction syndrome: This was defined, using the SOFA score, as the presence of ≥2 organs from the SOFA score being ≥3 in 24 hours. *PRBC*, packed red blood cells. *TBI*, traumatic brain injury (AIS Head and Neck ≥3).[6]

Table 2. Pneumonia microbiological characteristics

Organism	Number of Episodes
Staphylococcus aureus (MSSA)	6
Candida albicans	5
Klebsiella pneumonia	4
Haemophilus Influenza	4
Enterobacter cloacae	3
Escherichia coli	3
Acinetobacter baumannii	3
Klebsiella oxytoca	2
Staphylococcus aureus (MRSA)	1
Serratia marcescens	1
Pseudomonas aeruginosa	1

Pneumonia was diagnosed based on CDC criteria. Listed above, in descending order of frequency are all the organisms cultured and reported in a semi-quantitative manner from non bronchoscopic lower respiratory tract aspirates of patients diagnosed with pneumonia that, in conjunction with the microbiologists, were deemed plausibly to play a pathogenic role in the pneumonic process. A number of organisms were cultured from individual patients hence the number of positive cultures is in excess of the number of episodes of pneumonia. Candida albicans was never grown as the sole organism in any patient. MRSA, methicillin -resistant staphylococcus aureus. MSSA, methicillin -sensitive staphylococcus aureus.

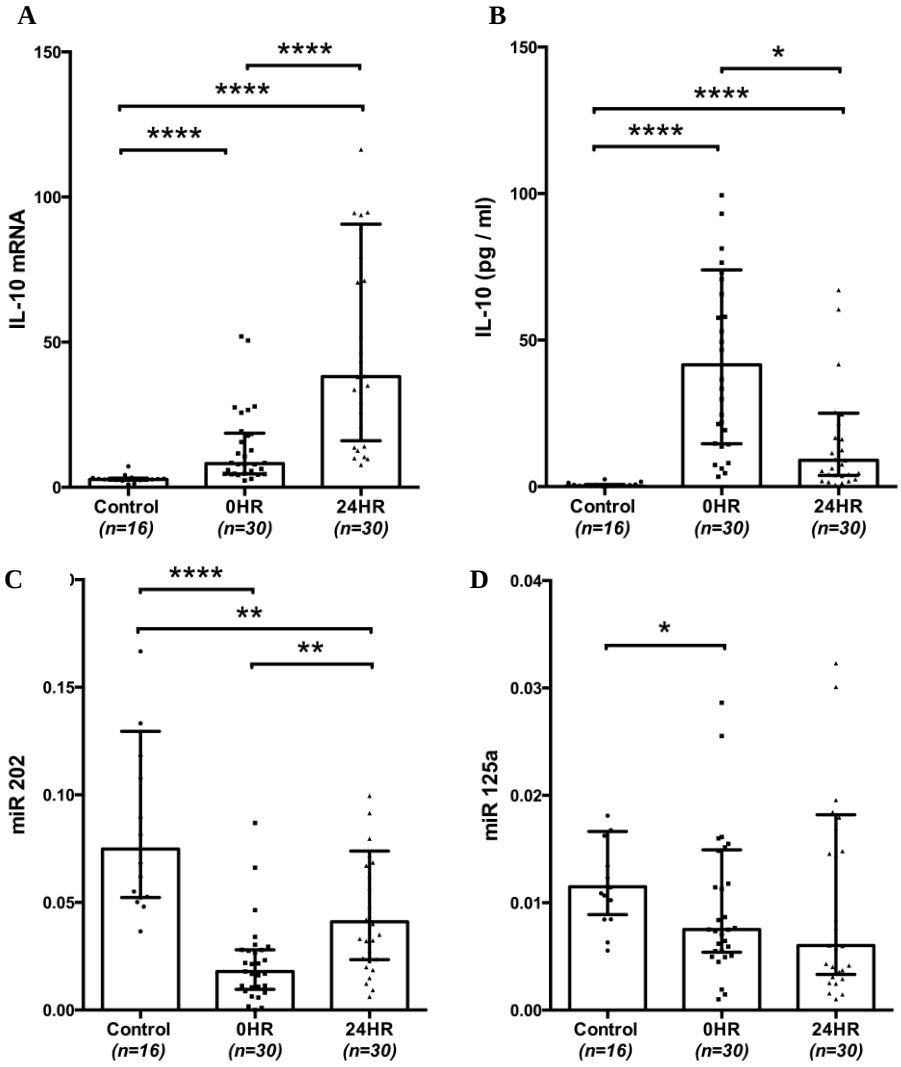

Figure 1. IL-10 mRNA levels, IL-10 protein levels and levels of miRs targeting IL-10; following severe trauma: Each graph represents levels in healthy controls, trauma patients at 0hr (admission) and 24hr. *Graph A*, IL-10 mRNA in whole blood; *Graph B*, IL-10 protein in plasma; *Graph C*, IL-10 regulating miR-202; *Graph D*, IL-10 regulating miR-125a. Graphs represent median and interquartile range. Graphs A, C and D are expressed as a relative quantification ratio between the candidate and the reference genes. Graph B is expressed as detected concentration via ELISA (pg / ml). *P*-values: *<0.05; **<0.01; ***<0.001; ****≤0.0001.

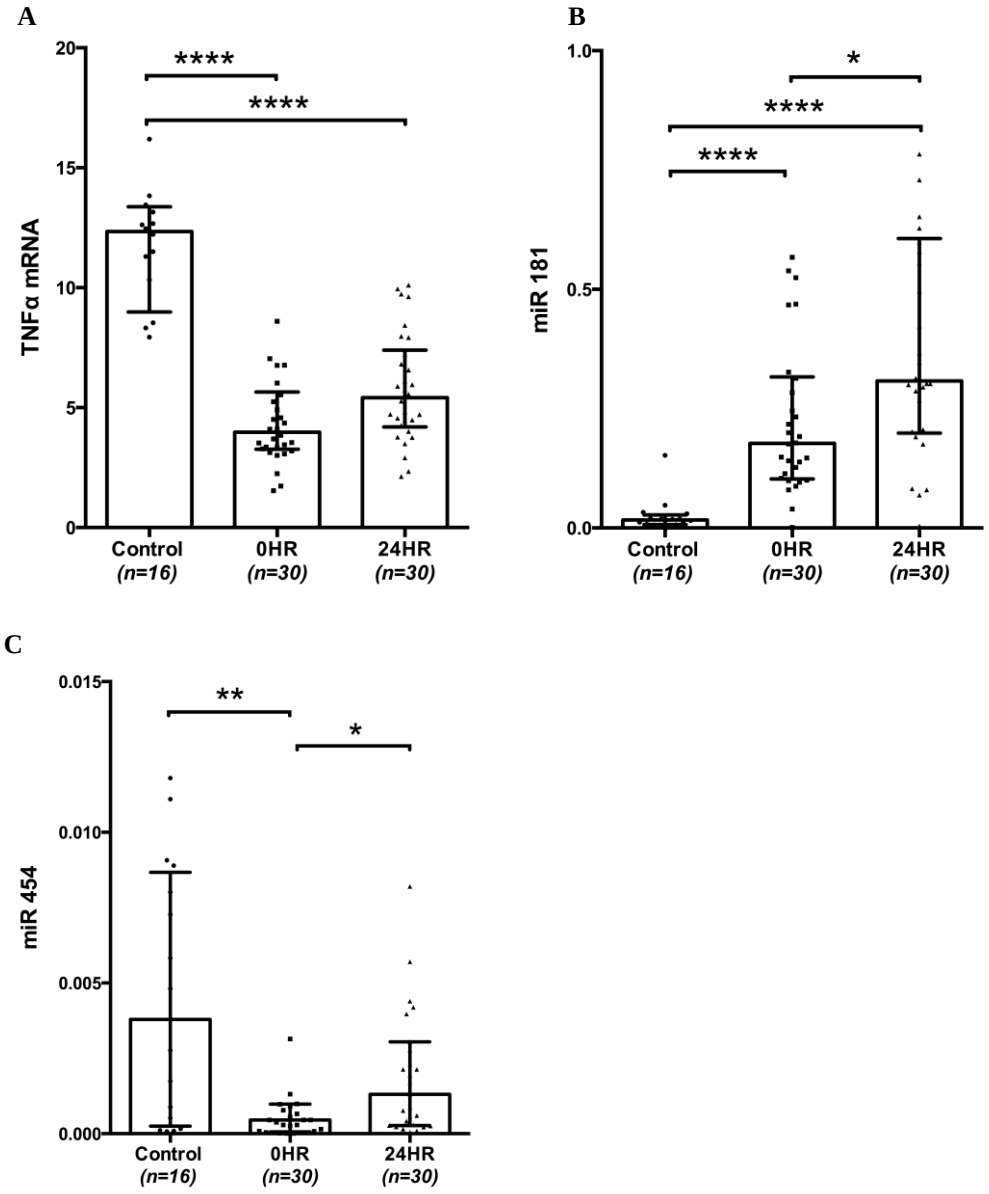

Figure 2. TNF-α mRNA levels and levels of miRs targeting TNF-α; following severe trauma: Each graph represents levels in healthy controls, trauma patients at 0hr (admission) and 24hr. *Graph A*, TNF-α mRNA in whole blood; *Graph B*, TNF-α regulating miR-181; *Graph C*, TNF-α regulating miR-454. Graphs represent median and interquartile range. Graphs A-C are expressed as a relative quantification ratio between the candidate and the reference genes. *P*-values: *<0.05; **<0.01; ***<0.001; ****≤0.0001.

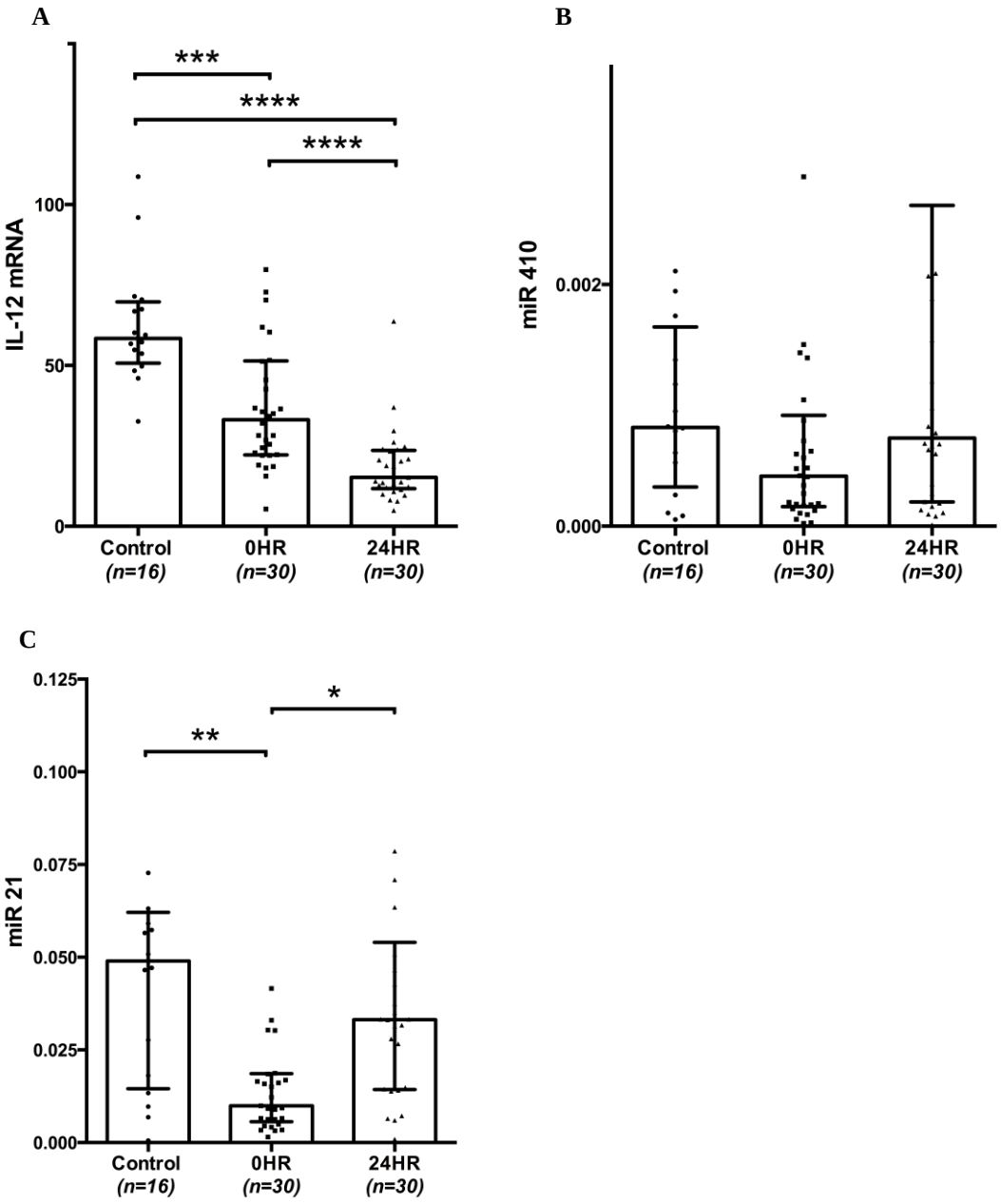

Figure 3. IL-12 mRNA levels and levels of miRs targeting IL-12; following severe trauma: Each graph represents levels in healthy controls, trauma 0hr (admission) and 24hr. *Graph A*, IL-12 mRNA; *Graph B*, IL-12 regulating miR-410; *Graph C*, IL-12 regulating miR-21. Graphs represent median and interquartile range. Graphs A-C are expressed as a relative quantification ratio between the candidate and the reference genes. *P*-values: *<0.05; **<0.01; ***<0.001; ****≤0.0001.

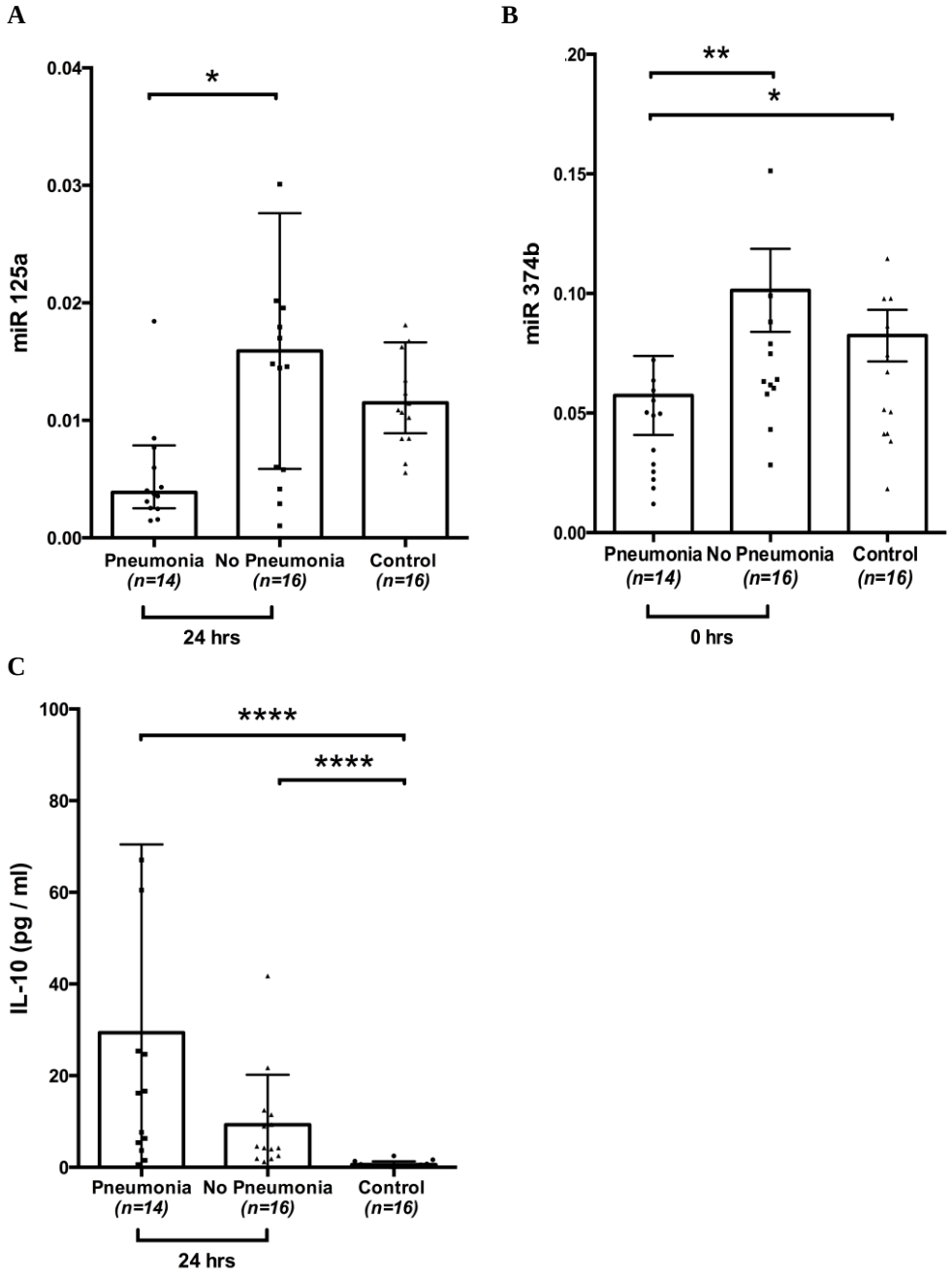

Figure 4. Associations between miRs targeting IL-10, IL-10 protein and pneumonia: Each graph represents mediator levels in severely injured patients developing pneumonia, those remaining free of pneumonia and healthy controls. *Graph A*, miR125a assayed at 24hr in pneumonia vs non pneumonia patients and normal healthy controls; *Graph B*, miR374b assayed at 0hr (admission) in pneumonia vs non pneumonia patients and normal healthy controls; *Graph C*, IL-10 plasma quantified at 24hr in pneumonia vs non pneumonia patients and normal healthy

controls. Graphs represent median and interquartile range. Graphs A & B are expressed as a relative quantification ratio between the candidate and the reference genes. Graph C is expressed as detected concentration via ELISA (pg / ml). *P*-values: *<0.05; **<0.01; ***<0.001; ****≤0.0001.

Supplementary Table 1. Criteria used for defining pneumonia

Radiology	**Two or more serial chest radiographs with at least 1 of the following:** • New or progressive and persistent infiltrate, consolidation or cavitation.
Signs & Symptoms	**For any patient, at least 1 of the following:** • Fever (>38°C or >100.4°F) with no other recognized cause. • Leukopenia (<4000 WBC/mm3) or leukocytosis (≥12,000 WBC/mm^3). • For adults ≥70 years old, altered mental status with no other recognized cause. **And at least 2 of the following:** • New onset of purulent sputum or change in character of sputum or increased respiratory secretions or increased suctioning requirements. • New onset or worsening cough, or dyspnea, or tachypnea. • Rales or bronchial breath sounds. • Worsening gas exchange (e.g., O_2 desaturations [eg, $P_aO2/FiO2$ ≤240], increased oxygen requirements, or increased ventilator demand).

Pneumonia can be diagnosed either by radiological criteria or by signs and symptoms. These criteria have been taken from reference 12.

Supplementary Table 2. miR targets

Gene	miRNA	mirSVR Score	Sequence Alignment
IL10	hsa-miR-374b	-1.3235	Position 205-212 of IL10 3' UTR 5' ...UAGAAAGAAGCCCAAUAUUAUAA...
			hsa-miR-374b 3' GUGAAUCGUCCAACAUAAUAUA
	hsa-miR-125a-3p	-1.3235	Position 235-241 of IL10 3' UTR 5' ...CAAUAUUUAUUAUUUUCACCUGU...
			hsa-miR-125a-3p 3' CCGAGGGUUCUUGGAGUGGACA
	hsa-miR-202	-1.0964	Position 139-146 of IL10 3' UTR 5' ...UAUUUAUUACCUCUGAUACCUCA...
			hsa-miR-202 3' AAGGGUACGGGAUAUGGAGA
TNFα	hsa-miR-181a	-1.277	Position 500-507 of TNF 3' UTR 5' ...UUAUUUAUUUACAGA--UGAAUGUA...
			hsa-miR-181a 3' UGAGUGGCUGUCGCAACUUACAA
	hsa-miR-301a	-1.1996	Position 451-457 of TNF 3' UTR 5' ...UCCCUCUAUUUAUGUUUGCACUU...
			hsa-miR-301a 3' CGAAACUGUUAUGAUAACGUGAC
	hsa-miR-454	-1.1996	Position 451-457 of TNF 3' UTR 5' ...UCCCUCUAUUUAUGUUUGCACUU...
			hsa-miR-454 3' UGGGAUAUUCGUUAUAACGUGAU
IL12	hsa-miR-410	-1.3439	Position 326-332 of IL12 3' UTR 5' ...UUUUAAAAUAUUUAUUUAUAUAA...
			hsa-miR-410 3' UGUCCGGUAGACACAAUAUAA
	hsa-miR-21	-1.3231	Position 256-263 of IL12 3' UTR 5' ...GAAGGGCAAAUAUUUAUAAGCUA...
			hsa-miR-21 3' AGUUGUAGUCAGACUAUUCGAU
	hsa-miR-590-5p	-1.3231	Position 256-263 of IL12A 3' UTR 5' ...GAAGGGCAAAUAUUUAUAAGCUA...
			hsa-miR-590-5p 3' GACGUGAAAAUACUUAUUCGAG

miRNAs predicted to target cytokines of interest based on microrna.org and TargetScan databases. The top 3 miRs based on mirSVR scores were selected for further analysis.

Supplementary Table 3. Univariate associations between miRNAs and clinical, demographic and injury specific variables.

	miR125a 0HR	**miR125a 24HR**	**miR202 0HR**	**miR202 24HR**	**miR374 0HR**	**miR374 24HR**
Age	0.63, 0.0005	0.2, 0.04	0.11, 0.06	0.33, 0.02	0.13, 0.05	0.07, 0.08
Scene SBP	0.46, 0.02	0.81, 0.002	0.19, 0.07	0.89, 0.0008	0.66, 0.008	0.35, 0.04
ED SBP	0.33, 0.03	0.46, 0.02	0.09, 0.10	0.46, 0.02	0.32, 0.03	0.37, 0.03
BE 0HR	0.96, 0.00008	0.16, 0.07	0.31, 0.03	0.58, 0.01	0.65, 0.008	0.87, 0.001
ISS	0.33, 0.03	0.73, 0.004	0.38, 0.02	0.40, 0.02	0.82, 0.001	0.87, 0.001
Sex*	0.6	0.85	0.07	0.09	0.68	0.64
Scene GCS	0.03, 0.15	0.18, 0.06	0.28, 0.04	0.58, 0.01	0.61, 0.009	0.50, 0.01
PRBC (units) in 24H	0.53, 0.01	0.65, 0.007	0.003, 0.28	0.004, 0.26	0.08, 0.10	0.12, 0.09
FFP (units) in 24H	0.39, 0.02	0.79, 0.002	0.05, 0.13	<0.0001, 0.47	0.005, 0.25	0.03, 0.18
Platelets (pools) in 24H	0.37, 0.02	0.75, 0.003	0.06, 0.12	0.0005, 0.36	0.04, 0.14	0.05, 0.14
Crystalloid (ml) in 24H	0.90, 0.005	0.42, 0.02	0.85, 0.001	0.82, 0.001	0.69, 0.005	0.21, 0.06
Colloid (ml) in 24H	0.63, 0.008	0.49, 0.01	0.47, 0.01	0.04, 0.14	0.01, 0.20	0.03, 0.17
AIS Head & Neck	0.24, 0.04	0.55, 0.01	0.80, 0.002	0.08, 0.10	0.08, 0.10	0.50, 0.01
AIS Face	0.73, 0.004	0.37, 0.02	0.66, 0.007	0.64, 0.007	0.42, 0.02	0.65, 0.008
AIS Thorax	0.01. 0.20	0.95, 0.0001	0.45, 0.02	0.86, 0.0001	0.65, 0.007	0.52, 0.01
AIS Abdomen & Pelvis	0.15, 0.07	0.96, 0.0001	0.76, 0.003	0.54, 0.01	0.19, 0.06	0.43, 0.02
AIS Extremity	0.19, 0.06	0.49, 0.01	0.14, 0.07	0.24, 0.04	0.06, 0.12	0.13, 0.08
Blunt vs. Penetrating	0.75	0.91	0.05	0.55	0.56	1

When two numbers as presented in a cell these represent an r^2 value and associated *P*-value. When one number is present this represents the p value of a Wilcoxon Test between two categorical variables. *AIS*, Abbreviated injury score. *BE*, Base Excess. *ED*, Emergency Department. *FFP*, Fresh Frozen Plasma. *GCS*, Glasgow Coma Score. *ISS*, Injury Severity Score. *PRBC*, packed red blood cells. *SBP*, Systolic Blood Pressure.